Analytical Methods for Assessing Exposure to 4-Aminobiphenyl Based on Protein Adduct Formation

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Past studies with animals have demonstrated that 4-aminobiphenyl (ABP) administration results in the formation of appreciable amounts of adducts between the carcinogen and both serum albumin and hemoglobin. The hemoglobin adduct is relatively stable in vivo, but may be readily hydrolyzed in vitro to regenerate ABP. The formation of this adduct reflects a mixed-function oxidase-mediated metabolic pathway operating directly on the amine. The predominant albumin adduct, 3-(tryptophan-N1-yl)-4-acetylaminobiphenyl, reflects the contribution of N-acetyltransferase activity as well as mixed function oxidase activity to the overall metabolism. The simultaneous measurement of these two different adducts thus offers an opportunity to investigate the role of both ABP and acetylator phenotype in bladder carcinogenesis. An analytical method, using gas chromatography coupled with electron capture detection, was developed to quantitate ABP adducted to hemoglobin.

The idea that accurate surveillance of exposure to carcinogens could be accomplished by measurement of the covalent adducts formed between the carcinogen and proteins was first proposed almost a decade ago. In 1974, Ehrenberg et al suggested that the reaction products of alkylating agents with circulating proteins might be useful historical records of the exposure of a person to such genotoxic agents.[1] The formation of carcinogen-protein adducts as a result of carcinogen administration to experimental animals has now been measured for more than 30 compounds, including directly acting agents as well as those requiring metabolic activation.[2] The essential premises of the approach have been demonstrated. The field is now ready to move forward to application to human populations with occupational or other exposure to genotoxic substances.

As an alternative to dosimetry based on measured or estimated ambient levels of a carcinogen in air, food, or water, protein adduct-based dosimetry offers several significant advantages. In addition to greater accuracy and the detection of unsuspected exposures, this method provides better measure of the effective dose. Many chemicals are handled by metabolic pathways that lead to detoxification as well as activation. Only the latter are toxicologically significant. Finally, protein adduct-based dosimetry will provide measurements with more relevance to the individual subjects because they will reflect such individual differences as metabolism.

Hemoglobin and albumin are the two most promising proteins for dosimetry. Hemoglobin is abundant—the blood of an adult male contains about 700 g of hemoglobin—and it contains amine, sulfhydryl, and aromatic groups with which carcinogens can react. Also, the lifetime of hemoglobin in the red blood cell is about 120 days. In the absence of accelerated destruction, then, the circulating level of a carcinogen-modified hemoglobin would reflect the integral of the carcinogen dose over a period of several months. Albumin is also abundant and reactive, has a half-life in blood of 20 to 24 days, and is synthesized in the hepatocyte, a site where many carcinogens are metabolized to their reactive forms.

The relations between the different stages of exposure, metabolic, and physiological processing, and ultimate biological reaction are depicted in Fig. 1. Meas-

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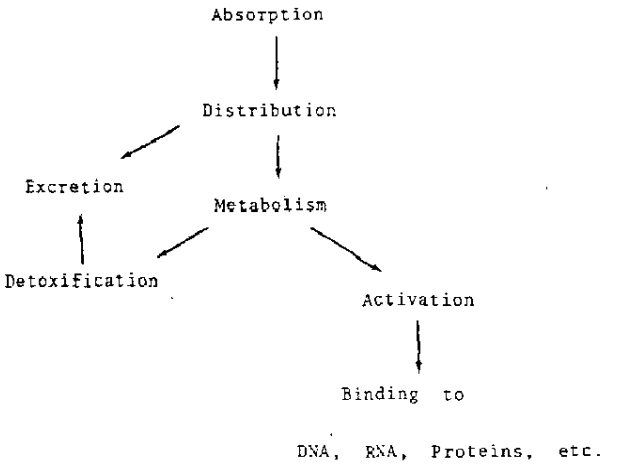

Fig. 1. Fate of carcinogens in vivo.

urements of DNA adducts and mutation or other genetic defects have been more widely applied to biological monitoring than has measurement of protein adducts. However, this latter measurement could greatly aid the interpretation of results from the other procedures, providing important and useful information on the extent of exposure integrated over a period of days, weeks, or months. In the case of mutation, for example, the results may reflect the exposure experienced over a lifetime. DNA adducts, although potentially more significant, may reflect exposure over only a few days. Half-lives for DNA adducts can be expected to vary greatly, from perhaps half a day to weeks, depending on chemical stability and the state of specific repair systems. To the best of our knowledge, there are no active repair systems for hemoglobin or albumin, which greatly simplifies the interpretation of adduct levels on these proteins compared with DNA adduct levels. Thus, protein adducts represent a new form of chemical dosimetry which may be used with DNA adducts and mutation to represent both the past and recent history of an individual's exposure to genetically toxic substances.

Among the various carcinogens that have been studied with regard to their blood protein adduct-forming properties are direct-acting alkylating agents (methyl methanesulfonate, methyl bromide and chloride, ethylene and propylene oxide, and N-nitroso-methylurea and N-nitroso-ethylurea); aromatic amines (4-aminobiphenyl, 4-aminostilbene, benzidine, aniline); and several other compounds with nothing more in common than that they require metabolic activation (aflatoxin, benzo(a)pyrene, chloroform). Farmer et al provide a more complete listing.[2] With one exception, all experiments have been performed in vivo with experimental animals or in vitro with isolated blood. Only ethylene oxide adducts have been determined in humans, for whom the source of exposure was occupational (sterilizing equipment used in hospitals).[3] Both directly acting and metabolically activated carcinogenic alkylating agents are well represented on the list.

The binding of carcinogens to blood proteins may be exploited for the purpose of dosimetry if several conditions have been satisfied: (1) the dose-response relationship must be predictable and known; (2) the amount of adduct formed must be great enough to be detectable by available means; and (3) an unambiguous analytical method must exist for quantifying adduct levels.

In this report, we review some earlier results from our work with the human bladder carcinogen 4-aminobiphenyl (ABP), research designed to address the first two points, and details of an analytical method for aminobiphenyl-hemoglobin adducts. The method described is sufficiently sensitive to measure the levels that are expected to arise from exposures great enough to have oncological significance.[4] In addition, some preliminary results are presented from research directed toward the development of immunoassays for the carcinogen.

Background

Administration of tritium-labeled ABP to rats resulted in the accumulation of a large fraction (5% to 10%) of the dose in the blood compartment. This fraction was invariant over a range of doses from 500 ng/kg to 5 mg/kg. Detailed analysis of the distribution of radioactivity demonstrated that more than 90% of it was covalently bound to the hemoglobin in the red cells. Nearly all of this 90% consisted of a unique modification at a single site in the protein: the β-93 cysteine residue. The remainder, not more than a few percent, was not identified. The β-93 cysteine adduct was shown to be formed from 4-hydroxylaminobiphenyl, a major initial metabolite of ABP in the liver. It was highly persistent, being removed from the blood at a rate similar to that of the removal of the red blood cells, indicating that there are no active repair mechanisms for this lesion. With chronic dosing, levels of this adduct accumulated until they reached a plateau, after roughly 60 days, that was about 30 times as great as the level produced by a single dose.

In the plasma compartment, albumin had accumulated a modest fraction of the dose, from 0.1% to 0.3%, depending on the effort expended on purification. In fact, much of this apparent binding was found to be noncovalent in nature. However, it was observed that one covalent adduct had formed with the one tryptophan residue, and this accounted for 0.02% of the administered dose. The ABP residue in this adduct possessed an N-acetyl group, suggesting that N-acetylation in the liver had preceded the cytochrome P-450 mediated activation to a reactive species. The tryptophan adduct, like the cysteine adduct in hemoglobin, was not repaired, and thus persisted with the protein until normal turnover of the protein effected its removal.

Analysis of Adducts by Gas Chromatography

Binding of ABP to the cysteine residue in hemoglobin results in an adduct that may easily be hydrolyzed to regenerate the originally administered carcinogen. Hydrolysis conditions are considerably milder than those necessary to cleave the peptide bond. We have taken

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advantage of this sensitivity to cleavage to develop a highly sensitive and selective analytical technique that does not depend for its success on the use of radioisotopes, and thus can be used with blood specimens obtained from human subjects.

The first step in the analysis of bound ABP in a blood specimen is the preparation of a hemolysate. This is accomplished by standard techniques that may vary slightly depending on the species from which blood is acquired. An internal standard, 4′-fluoro-ABP, is added to the hemolysate. This compound has physical and chromatographic properties nearly identical to those of ABP and is used to quantify the amount of ABP in the sample. The hemolysate is then made basic and incubated briefly at about 50 degrees. The ABP released from the cysteine adduct and the added standard are then extracted into hexane from the basic hemolysate, into acid from the hexane, and finally into hexane again from the acid layer after neutralization. The amines are then derivatized with a perfluorinated acylating agent in the hexane solution, and the solution is concentrated with a rotary evaporator and is ready for injection into the gas chromatograph.

For chromatography, it is essential to employ capillary columns to achieve both adequate resolution of the mixture and sensitivity. An electron capture detection system is also used in order to attain sufficient sensitivity. Considerable care must also be given to other aspects of the system such as purity of gases.

With our system, we are able to accurately determine adduct levels of 0.1 ng in 10 mL of whole blood. We developed a system with such sensitivity because of our interest in biochemical epidemiology of cigarette smoking. Exposure to ABP from cigarette smoking is estimated as no more than 200 ng/day based on reported values of about 2 to 5 ng per cigarette.[5] The rat model predicts that chronic human exposure to 200 ng/day will result in adduct levels of about 1 ng/10 mL blood. Dosimetry of occupational or other probable exposures will almost certainly involve higher adduct levels, which can now be performed routinely. Lower adduct levels (<0.1 ng/10 mL blood) might be measurable, but their oncological significance is doubtful because the corresponding exposure would be equivalent to only a few milligrams of ABP in a lifetime.

Hemoglobin-Bound 4-Aminobiphenyl in Untreated Subjects

Recently, we have been applying the system just described to the examination of not only blood obtained from rats dosed with ABP, but also specimens from both human subjects and untreated rats. Quantitation of the binding of an administered dose by the gas chromatography method agreed well with results obtained in earlier studies in which the administered ABP was tritium-labeled and the quantitation was by liquid scintillation counting. We were able to examine the binding of doses as low as 50 ng/kg and found that the fraction of dose bound to hemoglobin was as great as with larger doses. Most interesting, however, was the observation of ABP-hemoglobin adducts in the absence of any administered dose.

In the rat, the background level of hemoglobin adduct varied from about 0.4 ng/10 mL blood to nearly 2 ng. The background level has been observed in all of the more than 15 animals thus far examined, with animals coming from several cohorts. The observed values cor-

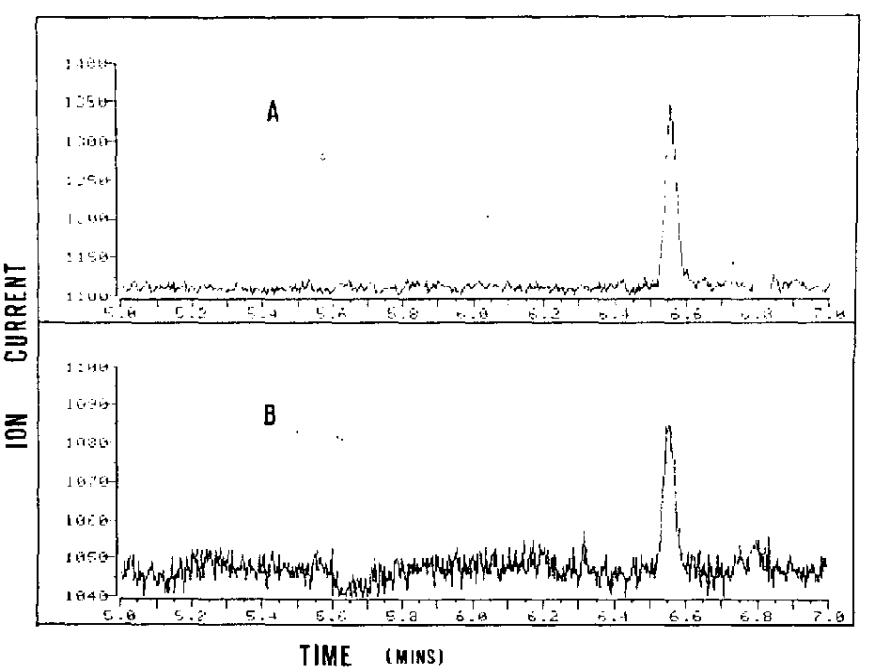

Fig. 2. Single ion mass chromatograms at m/z 315 of (A) 100 pg N-pentafluoropropionyl-4-aminobiphenyl and (B) extract from hydrolyzed rat hemoglobin, derivatized with pentafluoropropionic anhydride. ABP had never been administered to the animals; its presence as a hemoglobin adduct is presumed to result from environmental exposure.

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respond to a chronic average dose rate of 1 to 5 ng/kg/day. In the human specimens, levels of from 0 to 0.25 ng/10 mL blood have been observed, values that are only about one-tenth of those observed in the rat.

Because identity of the retention time of a sample with that of a standard on a gas chromatograph is not sufficient evidence for the identity of a substance, we also examined a sample on a gas chromatograph coupled with a mass spectrometer. The ion current was monitored at the mass corresponding to the molecular weight of derivatized ABP, because the derivative does not fragment significantly, and the molecular ion is thus the most abundant. Chromatograms of standard ABP and the component extracted from hydrolyzed rat hemoglobin are illustrated in Fig. 2. The sample was obtained from the pooled blood from four rats to provide a sufficiently high concentration. Only one peak, having the retention time of the authentic material, was detected. We are thus confident that the peak in question is indeed ABP.

Development of Radioimmunoassay: Analysis of Acetylaminobiphenyl

The serum albumin adduct of ABP is of interest because it is a possible dosimeter for acetylated ABP. Several studies now point to a crucial role for acetylation in the risk for bladder cancer.[6-8] From these reports, it seems likely that acetylated metabolites are not responsible for initiation of bladder tumors, although they may be involved in the toxicity of ABP in other organs. In any event, the slow acetylator phenotype is positively correlated with increased bladder cancer risk. Thus, although it may be reasonable to estimate exposure to ABP from the levels of hemoglobin adduct, it may be possible to gain a better measure of the individual risk by simultaneously estimating the fraction of ABP that is acetylated and thus diverted from bladder tumor initiation.

The analytical approach developed for the hemoglobin adduct will not serve for the albumin adduct because of both an inadequate sensitivity and the fact that the albumin adduct cannot be made to regenerate ABP. An immunoassay, however, can have the necessary selectivity and sensitivity. The remainder of this discussion deals with our efforts to develop a good immunoassay.

A very effective antigen was prepared by coupling ABP to bovine γ-globulin or bovine serum albumin. The amine was treated with nitrous acid to form a diazonium ion which was allowed to react with the protein. Between 20 and 40 molecules of amine could be bound to each molecule of protein by this reaction. When mice were immunized with these antigens, a strong and rapid response was observed in seven out of eight animals. Only a single injection of 50 μg in complete Freund's adjuvant was necessary, followed after 3 weeks by a single booster in incomplete Freund's. Within 10 days of administration of the booster, sera were strongly positive when tested in a radioimmunoassay using tritium-labeled N-acetyl-4-aminobiphenyl and unlabelled ABP. Thus, it is clear that the epitope is not the azo-linkage used to bind ABP to protein to form an antigen, but rather the biphenyl portion of the molecule.

Conclusion

By means of the gas chromatographic system we have developed, hemoglobin-bound ABP can now be measured at levels resulting from chronic daily exposures to amounts as low as 10 to 20 ng. Studies are currently under way with human subjects with known smoking histories to determine if exposure to cigarette smoke, which is reported to contain ABP, significantly increases exposure to this carcinogen. It would also be of interest to examine subjects with occupational exposure to ABP. Although its industrial use has largely been curtailed since the discovery of its carcinogenicity, exposure may well result from its formation as an unwanted side product in the manufacture of other amines or dyes. Exposure in such cases could easily be greater than the nanogram amounts in cigarette smoke, with the result that epidemiological studies should be easier to interpret.

Immunoassay research on ABP adducts is still in the developmental stage. The eventual attainment of an immunoassay will permit the measurement of serum albumin adducts of the carcinogen, which are actually composed of the N-acetyl metabolite of ABP. With this additional measurement, it should be possible to refine the dosimetry so as to evaluate the role of N-acetylation in risk for bladder cancer.

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